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Research paper

Superoxide anion-induced pain and inflammation depends on TNFα/TNFR1 signaling in mice

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HIGHLIGHTS

- Superoxide anion induces pain and inflammation.
- Targeting TNFα/TNFR1 signaling reduces superoxide anion-induced pain/ inflammation.
- TNFα/TNFR1 signaling mediates superoxide anion-induced oxidative stress.
- Inhibiting NADPH oxidase and SOD mimicry reduce TNFα-induced pain and inflammation.
- Superoxide anion-induced pain and inflammation depends on TNFα/TNFR1 signaling.

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ABSTRACT

Inhibition of tumor necrosis factor-alpha (TNFα) and superoxide anion production reduces inflammation and pain. The present study investigated whether superoxide anion-induced pain depends on TNFα signaling and the role of superoxide anion in TNFα-induced hyperalgesia to clarify the interrelation between these two mediators in the context of pain. Intraplantar injection of a superoxide anion donor (potassium superoxide) induced mechanical hyperalgesia (0.5–5 h after injection), neutrophil recruitment (myeloperoxidase activity), and overt pain-like behaviors (paw flinching, paw licking, and abdominal writhings) in wild-type mice. Tumor necrosis factor receptor 1 deficiency (TNFR1-/-) and treatment of wild-type mice with etanercept (a soluble TNFR2 receptor that inhibits TNFα actions) inhibited superoxide anion-induced pain-like behaviors. TNFR1$^{-/-}$ mice were also protected from superoxide anion donor-induced oxidative stress, suggesting the role of this pathway in the maintenance of oxidative stress. Finally, we demonstrated that Apocynin (an NADPH oxidase inhibitor) or Tempol (a superoxide dismutase mimetic) treatment inhibited TNFα-induced paw mechanical hyperalgesia and neutrophil recruitment (myeloperoxidase activity). These results demonstrate that TNFα/TNFR1 signaling is important in superoxide anion-triggered pain and that TNFα/TNFR1 signaling amplifies the oxidative stress triggered by superoxide anion, which contributes to sustaining pain and inflammation.

1. Introduction

Targeting superoxide anion and tumor necrosis factor-alpha (TNFα) alleviate pain and inflammation [1,17,22]. Depending on disease context model, TNFα sensitizes nociceptive neurons acting indirectly via prostaglandin E_2 production and directly on nociceptive neurons through the tumor necrosis factor receptor 1 (TNFR1).

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This sensitization results in hyperalgesia [13]. TNFα also induces inflammation, neutrophil recruitment and activates superoxide-generating systems, which contribute to increasing oxidative stress and pain [5,6,22]. Furthermore, superoxide anion also contributes to pain and hyperalgesia [13,17]. In fact, superoxide anion up-regulates TNFR1 expression in nociceptive neurons and increases TNFα production [18,20]. It is not known, however, whether TNFα signaling represents a crucial step in pain transmission and hyperalgesia induced by superoxide anion. In this study, we aimed to describe the role of TNFα/TNFR1 during pain and inflammation induced in mice by a superoxide anion donor (potassium superoxide). Additionally, we evaluated the reciprocal contribution of superoxide anion during mechanical hyperalgesia induced by TNFα injection.

2. Materials and methods

2.1. Animals

C57BL/6 wild-type (WT) control mice and C57BL/6 TNFR1$^{-/-}$ mice were provided by University of São Paulo (São Paulo, Brazil). Behavioral tests were performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal experiments and procedures were conducted according to the International Association for Study of Pain (IASP) guidelines and with approval (process 71.2012.68) by the Ethics Committee of Universidade Estadual de Londrina.

2.2. Drugs and reagents

Potassium superoxide (KO_2) 96.5% was purchased from Alfa Aesar (Ward Hill, MA, USA). Phosphate buffered saline (PBS), ortho-dianisidine dihydrochloride, 4-nitrophenyl *N*-acetyl-β-D-glucosaminide, trichloroacetic acid (TCA), thiobarbituric acid (TBA), nitro blue tetrazolium (NBT), HTAB (Bromide, hexadecyltrimethylammonium), dihydrochloride orto-dianisidine, glutathione (GSH), EDTA sodium salt, ferric chloride hexahydrate, TPTZ (2,4,6-tripiridil-*s*-triazine), ABTS [2,2-azinobis (3-ethylbenzothiazoline-6-10 sulfonate), diammonium salt], Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-11 carboxylic acid), PMSF (phenylmethane sulfonyl fluoride), Tempol, and Apocynin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Stimuli and treatments

KO_2 stimulus was diluted in saline immediately before its use and administered by intraplantar (i.pl.) (30 μg, 25 μL) or intraperitoneal (i.p.) (1 mg, 100 μL) injection [8]. TNFα stimulus (100 ng, 25 μL of saline) was administered by i.pl. injection. Etanercept (1, 3 or 10 mg/kg, diluted in 100 μL of saline) treatment was given by i.p. route 48 h and 1 h before KO_2 stimulus. Apocynin (100 mg/kg, i.p., diluted in 100 μL of DMSO 1% in saline) treatment was administered 30 min before TNFα stimulus. Tempol (100 mg/kg, i.p., diluted in 100 μL of saline) was administered 40 min before TNFα stimulus. Pilot studies based the doses of stimuli and treatment [17].

2.4. Behavioral tests

The International Association for the Study of Pain (IASP) defines hyperalgesia as an increase of pain sensation caused by a stimulus that normally induces a nociceptive response. On the other hand, allodynia is defined as pain sensation caused by a stimulus that normally does not induce nociceptive response [21]. Mechanical stimuli elicit both hyperalgesia and allodynia in sensitized tissues. The electronic von Frey is characterized by a basal response to mechanical stimulus, indicating mechanical hyperalgesia is under evaluation differing from the classic von Frey filaments, which does not elicit a basal response, therefore, evaluating allodynia. Another difference between electronic von Frey and classic von Frey filaments is that results are given in g and log, respectively [8]. Mechanical hyperalgesia was evaluated with a hand-held force transducer (electronic anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip between 0.5–5 h after KO_2 (30 μg, 25 μL) or TNFα (100 ng, 25 μL) i.pl. injection. In this method, mice were placed in acrylic cages with wire grid floors, 15 min before testing. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The removal of the paw followed by clear flinching movement characterized the end point, and the apparatus recorded the intensity of pressure automatically. Mice were tested before (basal) and after treatment and stimuli, and the value for each interval was an average of three measurements. The results are expressed by delta (Δ) withdrawal threshold (in g), calculated by subtracting the mean measurements at the indicated time points after stimulus from the basal mean measurements [8,17]. The total number of paw flinches and time spent licking the paw were measured 0–30 min after i.pl. stimulus with KO_2 (30 μg, 25 μL). Abdominal writhings were measured 0–20 min after i.p. stimulus with KO_2 (1 mg, 100 μL). TNFα was not able to induce overt pain-like behaviors (paw flinches, paw licking, abdominal writhings) [17].

2.5. Myeloperoxidase (MPO) activity

Neutrophil recruitment to plantar skin was evaluated by MPO activity kinetic colorimetric assay [2]. Mice were terminally anesthetized 5 h after stimulus injection. Plantar skin samples were collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% HTAB and stored at −20 °C. MPO activity was determined at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). A standard curve of neutrophils was used. Results are presented as the number of neutrophils ($\times 10^4$) per mg of tissue.

2.6. TNFα levels

Plantar skin samples were collected 3 h after i.pl. stimulus with KO_2 (30 μg, 25 μL) and homogenized immediately in ice-cold PBS buffer containing protease inhibitor (1 mM PMSF, Sigma–Aldrich). TNFα levels were determined at 450 nm (Multiskan GO, Thermo Scientific) by enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. Results are expressed as picograms (pg) of TNFα per mg of tissue.

2.7. Superoxide production

Superoxide anion production was measured in plantar skin samples collected 3 h after i.pl. stimulus with KO_2 (30 μg, 25 μL) by the nitroblue tetrazolium (NBT) assay adapted to a microplate [18]. NBT reduction was measured at 600 nm (Multiskan GO, Thermo Scientific). Sample weighting was used for data normalization.

2.8. Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) assay was used to determine lipid peroxidation [1]. Plantar skin samples were collected 3 h after i.pl. stimulus with KO_2 (30 μg, 25 μL) and homogenized immediately in ice-cold KCl buffer (500 μL, 1.15% w/v). Malondialdehyde (MDA) levels, an intermediate product of lipid peroxidation, was determined in samples by the difference between the absorbance at 535 and 572 nm (Multiskan GO, Thermo Scientific). Results are presented as nmol of MDA per mg of tissue.

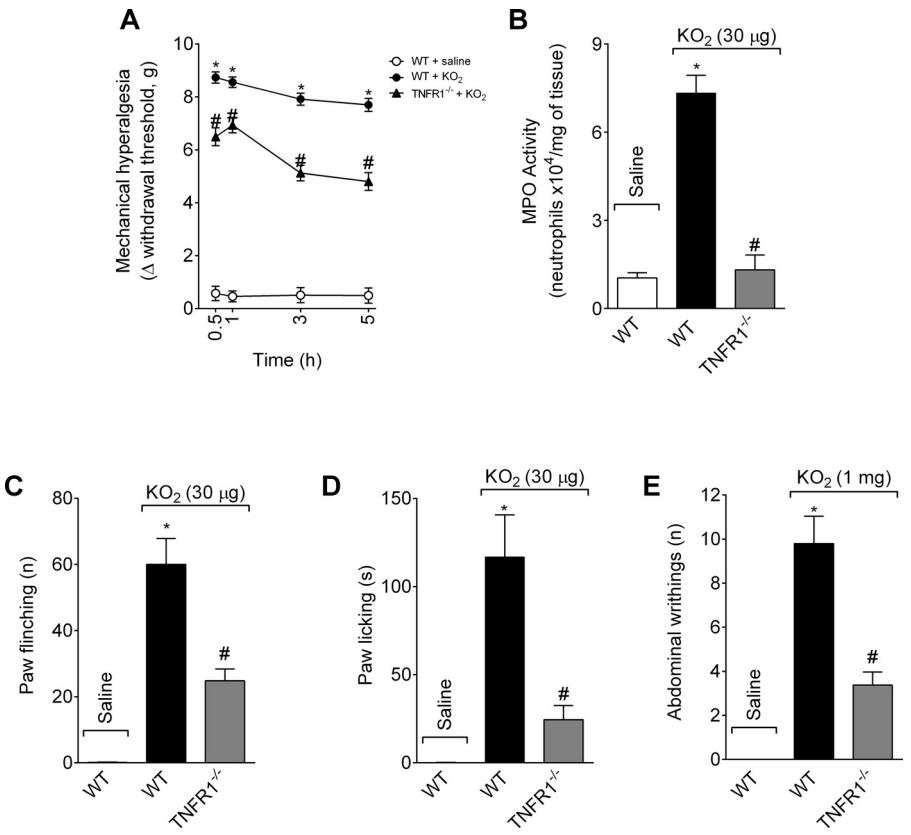

Fig.1. Superoxide anion-induced mechanical hyperalgesia, neutrophil recruitment, and overt pain-like behaviors depend on TNFR1 activation. Mice received i.pl. injection of 30 μg (A–D) or i.p. injection of 1 mg (E) of KO_2 (superoxide anion donor). Mechanical hyperalgesia (A) was determined at 0.5, 1, 3 and 5 h after the stimulus. Paw skin samples were collected at 5 h to determine myeloperoxidase activity (B). The number of flinches (C) and time spent licking (D) the paw were evaluated over 30 min. The total number of writhing (E) was evaluated over 20 min. Results are expressed as mean ± SEM ($n = 6$ per group per experiment, representative of two independent experiments). $^*p < 0.05$ vs. saline group $\#p < 0.05$ vs. KO_2 group (two-way repeated measure ANOVA followed Tukey's *post hoc* and for single time point One-way ANOVA followed Tukey's *post hoc*).

2.9. ABTS and FRAP assays

The ability of samples to resist oxidative damage was determined by its ABTS radical scavenging and ferric reducing (FRAP assay) properties. The tests were adapted to a 96-well microplate format as previously described [1]. Plantar skin samples were collected 3 h after i.pl. stimulus with KO_2 (30 μg, 25 μL) and homogenized immediately in ice-cold KCl buffer (500 μL, 1.15% w/v). The absorbance of ABTS and FRAP assays were measured at 730 and 595 nm (Multiskan GO Thermo Scientific), respectively and equated against a standard Trolox curve (0.02–20 nmol). Results are expressed as nmol of Trolox equivalent per mg of tissue.

2.10. GSH levels

Antioxidant levels of plantar skin were evaluated by measuring the levels of reduced glutathione (GSH), the most abundant non-enzymatic antioxidant of cells. Samples of plantar skin tissue were collected 3 h after KO_2 i.pl. stimulus (30 μg, 25 μL) and maintained at −80 °C for 48 h. GSH levels were measured as described previously [19], and the absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific). Results were equated against a standard GSH curve (0.02–20 nmol) and expressed as nmol of GSH per mg of plantar skin tissue.

2.11. Statistical analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group per experiment, representative of 2 independent experiments. Two-way repeated measure analysis of variance (ANOVA) followed by Tukey's *post hoc* was used when responses were measured at different times points after the stimulus injection. One-way ANOVA followed by Tukey's *post hoc* test was performed to compare the values at the indicated time points. Statistical differences were considered to be significant when $P < 0.05$.

3. Results

Superoxide anion-induced mechanical hyperalgesia, neutrophil recruitment, and overt pain-like behaviors depend on TNFR1 activation

Firstly, the role of TNFα in nociceptive neuron activation and sensitization induced by superoxide anion was investigated. For this purpose, we stimulated wild-type (WT) and TNFR1$^{-/-}$ mice with KO_2 and compared their responses regarding mechanical hyperalgesia, neutrophil recruitment, and overt pain-like behavior. Superoxide anion induced mechanical hyperalgesia at all evaluated intervals (0.5–5 h) in WT mice, while TNFR1$^{-/-}$ mice exhibited a significant reduction in the intensity of this effect (Fig. 1A). Neutrophil recruitment (MPO activity) to the plantar skin was fully inhibited in TNFR1$^{-/-}$ mice (Fig. 1B). TNFR1 deficiency also reduced superoxide anion-induced overt pain-like behaviors of paw flinching (Fig. 1C), paw licking (Fig. 1D), and abdominal writhings (Fig. 1E).

TNFα blockade by Etanercept treatment inhibits mechanical hyperalgesia, neutrophil recruitment, and overt pain-like behaviors induced by superoxide anion

The nociceptive and inflammatory responses induced by superoxide anion in WT mice treated with Etanercept (1–10 mg/kg, i.p.,

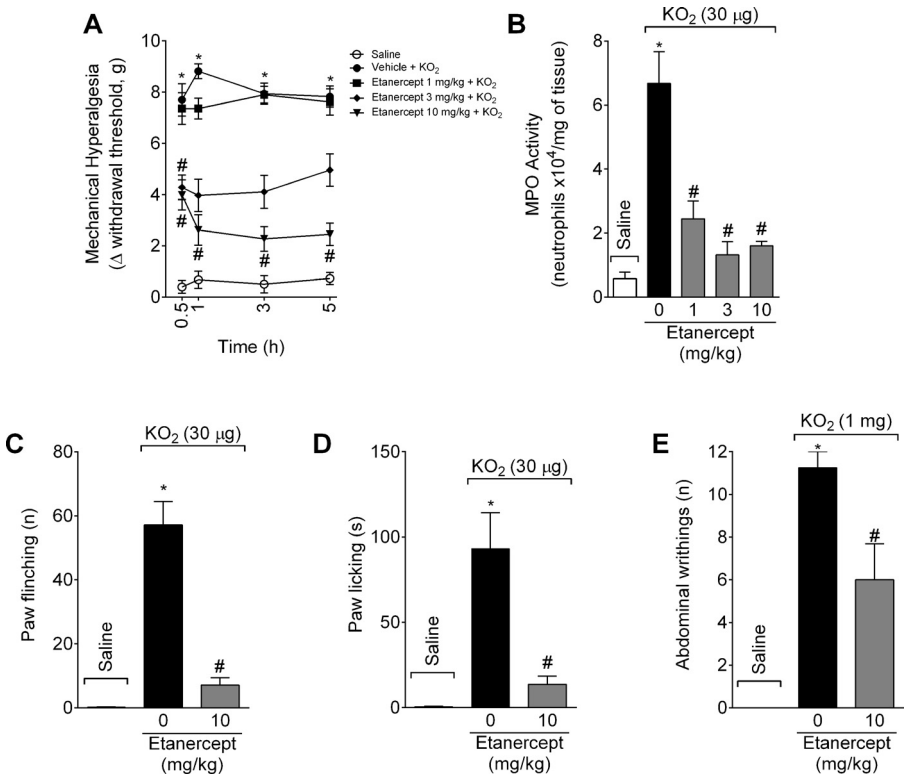

Fig. 2. TNFα blockade by Etanercept treatment inhibits in a dose-dependent manner the mechanical hyperalgesia, neutrophil recruitment, and overt pain-like behaviors induced by superoxide anion. Mice were treated with etanercept (1, 3 or 10 mg/kg, i.p.) 1 h before i.pl. injection of 30 μg (A–D) or i.p. injection of 1 mg (E) of KO_2. Paw skin samples were collected at 5 h to determine the myeloperoxidase activity (B). The number of flinches (C) and time spent licking (D) the paw were evaluated over 30 min. The total number of writhing (E) was evaluated over 20 min. Results are expressed as mean ± SEM ($n = 6$ per group per experiment, representative of two independent experiments). $^*p < 0.05$ vs. saline group $\#p < 0.05$ vs. KO_2 group (two-way repeated measure ANOVA followed Tukey's *post hoc* and for single time point One-way ANOVA followed Tukey's *post hoc*).

48 h and 1 h before KO_2 stimulus) were also tested. Etanercept is a soluble TNFR2 receptor that is therapeutically used to reduce the available TNFα diminishing the activation of membrane TNF receptors and consequent pro-inflammatory actions [10]. Etanercept treatment at the dose of 10 mg/kg reduced superoxide anion-induced mechanical hyperalgesia at all evaluated intervals (Fig. 2A). The dose of 3 mg/kg of etanercept was able to inhibit mechanical hyperalgesia at 0.5 h only (Fig. 2A). The dose of 1 mg/kg of etanercept did not alter superoxide anion-induced mechanical hyperalgesia (Fig. 2A). On the other hand, all three tested doses of etanercept were able to inhibit superoxide anion-induced neutrophil recruitment (MPO activity) in the plantar skin, without differences between the three doses (Fig. 2B). Ten mg/kg of etanercept reduced superoxide anion-induced overt pain-like behaviors of paw flinching (Fig. 2C), paw licking (Fig. 2D), and abdominal writhings (Fig. 2E).

Superoxide anion increases TNFα production and induces oxidative stress in a TNFR1-dependent manner

Plantar skin samples were collected 3 h after superoxide anion stimulus to measure TNFα production and oxidative stress. We found an increase in TNFα concentration (Fig. 3A) in plantar skin samples 3 h after superoxide anion injection, suggesting the role of this cytokine in the maintenance of mechanical hyperalgesia induced by superoxide anion. In line with this hypothesis, superoxide anion injection triggered an up-regulation of endogenous production of superoxide anion that was dependent on TNFR1 receptor activity (Fig. 3B). Moreover, oxidative stress induced by superoxide anion injection was also dependent on TNFR1 activity (Fig. 3C–E).

TNFα-induced mechanical hyperalgesia and MPO activity depend on enzymes that generate superoxide anion.

Next, we investigated the role of endogenous superoxide anion production in the mechanical hyperalgesia and neutrophil recruitment (MPO activity) induced by TNFα. TNFα induced mechanical hyperalgesia at all evaluated time points (0.5–5 h) after injection (Fig. 4A), which was inhibited in mice treated with an NADPH inhibitor (Apocynin) or a superoxide dismutase mimetic (Tempol) (Fig. 4A). Furthermore, Apocynin and Tempol were able to reduce TNFα-induced neutrophil recruitment (MPO activity) in the plantar skin (Fig. 4B).

4. Discussion

The present study shows that superoxide anion-induced pain and inflammation depend on TNFα production and TNFR1 activation. This signaling generates a positive feedback loop: superoxide anion induces TNFα production that, in turn, triggers the endogenous production of superoxide anion.

Superoxide anion and TNFα seem to be key mediators of this positive feedback loop as their inhibition was effective in reducing mechanical hyperalgesia and neutrophil recruitment. In fact, it has been demonstrated that superoxide anion represents a crucial mediator during TNFα processing into its active form [20,26].

Herein, we provide evidence that superoxide anion also mediates the hyperalgesia that is induced after TNFα production. The present findings suggest that superoxide anion can depolarize nociceptive neurons since it evoked overt pain-like behaviors.

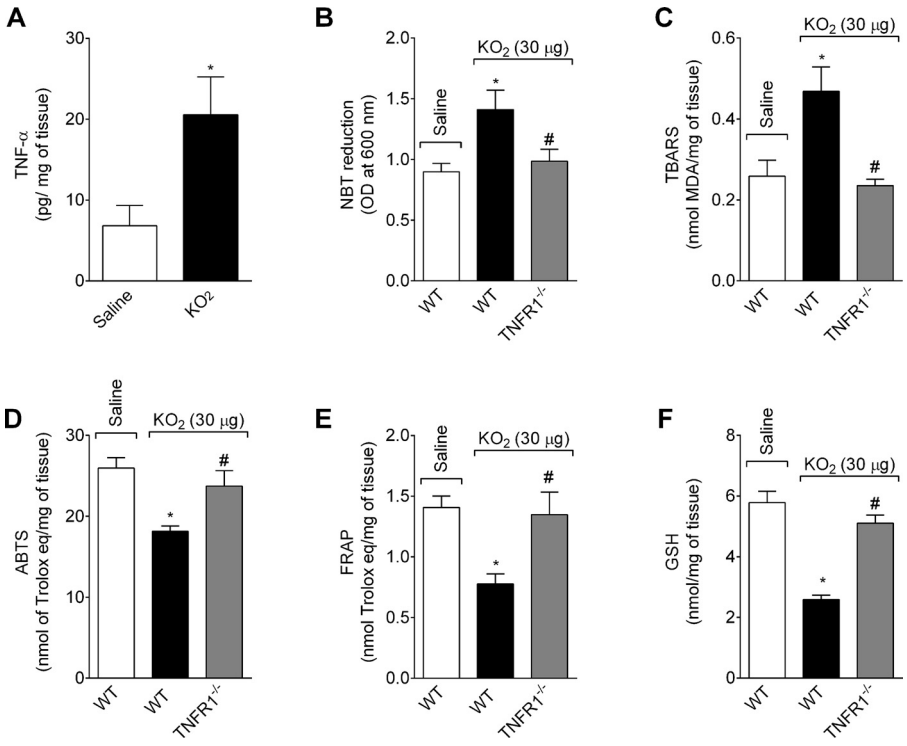

Fig. 3. Superoxide anion increases TNFα production and induces oxidative stress in a TNFR1-dependent manner. Mice received i.pl. injection of 30 μg KO_2. Paw skin samples were collected 3 h after stimulus to determine TNFα tissue concentration (A), superoxide anion production (NBT assay) (B), MDA formation (C) total antioxidant capacity (ABTS assay) (D), ferric reducing antioxidant power (FRAP assay) (E) and GSH concentration (F). Results are expressed as mean ± SEM ($n = 6$ per group per experiment, representative of two independent experiments). $^*p < 0.05$ vs. saline group $\#p < 0.05$ vs. KO_2 group (one-way ANOVA followed Tukey's *post hoc*).

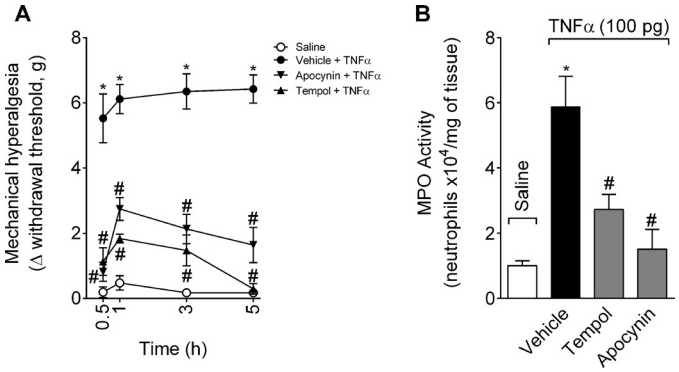

Fig. 4. TNFα-induced mechanical hyperalgesia and MPO activity depend on superoxide anion. Mice were treated with Apocynin (100 mg/kg, i.p., 30 min) or Tempol (100 mg/kg, i.p., 40 min) before i.pl. injection of 100 ng TNFα. Mechanical hyperalgesia (A) was determined at 0.5, 1, 3 and 5 h after the stimulus. Paw skin samples were collected at 5 h to determine myeloperoxidase (MPO) activity (B). Results are expressed as mean ± SEM ($n = 6$ per group per experiment, representative of two independent experiments). $^*p < 0.05$ vs. saline group $\#p < 0.05$ vs. TNFα group (two-way repeated measure ANOVA followed Tukey's *post hoc* and for single time point One-way ANOVA followed Tukey's *post hoc*).

In turn, TNFα plays a major role during neuronal sensitization, which is in line with previous works reporting that TNFα modulates the activity of sodium channels in nociceptive neurons [9,13]. Hence, we provided evidence that TNFα and TNFR1 are necessary to induce superoxide anion-triggered overt pain-like behaviors of paw flinching, paw licking, and abdominal writhings. Independent studies reported that reactive oxygen species and TNFα increase the excitability of nociceptive neurons through a p38 mitogen-activated protein kinase modulation of sodium channels activity [13,20,24,27–29]. However, the exact mechanisms by which superoxide anion uses TNFα/TNFR1 pathway in neurons to depolarize these cells is a topic that certainly deserves further investigation.

In addition to TNFR1, TNFR2 receptors are likely participating in superoxide anion-induced pain and inflammation since the treatment with etanercept induced a greater reduction of superoxide anion-induced mechanical hyperalgesia than the genetic deficiency of TNFR1. Etanercept is a soluble TNFR2 receptor that is therapeutically used to scavenge TNFα, thus preventing TNFα binding to membrane TNFR1 and TNFR2 receptors and the consequent cellular activation. The treatment with etanercept reduces pain in models of inflammation [3], cancer [4] and neuropathy [16]. TNFR2 and TNFR1 deficiency reduce inflammatory [7,14], cancer [11] and neuropathic pain [31]. Acute treatment with anti-TNFR1 and/or anti-TNFR2 antibodies also reduce nociceptive responses [12,30]. On the other hand, TNFR1/TNFR2 double deficiency enhanced pain sensitivity observed as bilateral orofacial pain following infraorbital nerve ligation, and chronic pain in arthritis induced by complete Freundís adjuvant injection in the knee joint followed by gastrointestinal challenge with intracolonic mustard oil injection (double hit model). Furthermore, TNFR1/TNFR2 double deficiency resulted in up-regulation of varied pro-inflammatory and hyperalgesic cytokines and chemokines [15,25]. In contrast, bone fibrosarcoma-induced tactile hypersensitivity was almost abolished in TNFR1/TNFR2 double deficient mice with reduced astrogliosis and increased spontaneous pain-like behavior and tumor growth. Genetic deficiency of one of TNF receptors did not affect bone cancer-induced tactile hypersensitivity, spontaneous pain-like behavior, spinal astrogliosis and tumor growth [11]. The injection of lung carcinoma cells in the paw induces thermal hyperalgesia with a major role of TNFR2 through enhanced expression of transient receptor potential vanilloid 1 (TRPV1) [4]. In chronic constriction injury-induced neuropathic pain, TNFR1 deficiency reduced thermal hyperalgesia, and TNFR1 or TNFR2 deficiency reduced mechanical and cold

allodynia [23]. Therefore, targeting TNFR1 and/or TNFR2 may present beneficial and/or detrimental effects depending on disease context.

5. Conclusions

TNFα/TNFR1 signaling contributes significantly to superoxide anion-induced inflammation and pain. There is a positive feedback loop in which superoxide anion induces TNFα production, which in turn induces superoxide anion-mediated inflammation and pain. This study contributes to understanding the inflammatory and nociceptive interaction between superoxide anion and TNFα suggesting that targeting TNFα reduces inflammatory diseases exhibiting superoxide anion-dependent mechanisms, and vice-versa.

Conflict of interest

The authors declare no conflict of interest.

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